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Microbial processing of sedimentary organic matter at a shallow LTER site in the northern Adriatic Sea: an 8-year case study

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Abstract

Benthic prokaryotes are the key-players in C-cycling at the sediment-seawater interface, one of the largest biologically active interfaces on Earth. Here, microbial-mediated processes, such as the degradation of organic matter and the incorporation of mobilized C into microbial biomass, depend on several factors such as environmental temperature and substrate availability, especially in shallow sediments at mid-high latitudes where seasonal fluctuations of these variables occur. In the present study, four degradative activities (β-glucosidase, lipase, chitinase and aminopeptidase), Heterotrophic C Production (HCP), Total Organic C (TOC), Total Nitrogen (TN) and Biopolymeric C (BPC) were investigated seasonally from April 2010 to April 2018 in the surface sediments of a shallow Long-Term Ecological Research (LTER) station of the northern Adriatic Sea. Significant temperature-dependences were described by Arrhenius-type equations for HCP and each of the degradative activities tested with the exception of aminopeptidase. The relatively low apparent Activation Energies suggested that these microbial-mediated processes were enhanced by the availability of palatable substrates over the study period. Nevertheless, a clear and tight dependence from such substrates was detected only for aminopeptidase, the most pronounced degradative activity observed.

TN was identified by the stepwise multiple regression analysis as the environmental variable that mainly drove this exoenzymatic activity. Enhanced aminopeptidase rates mirrored peaks of TN that seemed, in turn, linked to the seasonal proliferation of benthic microalgae. By supplying prokaryotes with promptly available substrates, these autotrophs, represented mainly by diatoms, seemed to play an important role in the C-cycling regulation at the studied LTER station.

Keywords

Benthic prokaryotes; exoenzymatic activities; Heterotrophic C Production; temperature; Total Nitrogen; Long-Term Ecological Research; LTER

Introduction

Collectively termed prokaryotes, bacteria and archaea play a pivotal role in C-cycling and geochemical processes as the main elements responsible for the degradation of organic matter and the incorporation of mobilized C into microbial biomass. In the marine environment, the majority of organic matter is, in fact, in a polymerized form that cannot be assimilated directly by the prokaryotic cells since only small molecules (<600 Da), such as aminoacids and monosaccharides, can pass through the membrane due to the activity of permeases. In order to make the organic matter available, prokaryotes induce the production of non-cytoplasmatic enzymes that hydrolyze polymers and oligomers into assimilable monomers (Chróst 1992). Although these active molecules can be cell-bound or cell-free (Baltar 2018), in the present study both kinds are inclusively referred to as exoenzymes. Their targets are specific chemical bonds that link the subunits that form organic polymers (i.e. sugars, peptides, nucleotides, structural and storage polysaccharides). The end-up products of exoenzymatic reactions are low molecular weight compounds that are ultimately incorporated into prokaryotic cells and used for growth.

Although both exoenzymatic activities and organic matter uptake (Heterotrophic C Production – HCP) are known for being influenced by several environmental variables (e.g. temperature, pH, salinity, availability of substrates, etc.), it is difficult to discriminate between the influence exerted by each of them and to evaluate any synergistic or antagonistic effect. Among the several variables that may influence microbial dynamics, the role of temperature and substrate availability aroused the interest of scientists for years (Pomeroy and Wiebe 2001, Calvo-Díaz et al. 2014, Lønborg et al. 2016). While some studies investigated only the effect of temperature on microbial C uptake (e.g. Lønborg et al. 2016), others tried to relate the observed microbial metabolic changes to both of them. For instance, Calvo-Díaz and co-authors (2014) measured bacterial production over two annual cycles in temperate coastal waters (southern Gulf of Biscay) and detected an overall switch from a more pronounced control exerted by resource availability in summer-early autumn samplings to a more prominent effect of temperature during winter and spring. Overall, these kinds of studies highlighted a variable susceptibility of bacterioplankton to both temperature and substrate availability, making quite difficult the extrapolation of any general description about their interaction (Pomeroy and Wiebe 2001).

To date, the majority of investigations pertaining to this issue have been carried out on bacterioplankton while organic matter processing in the sediments remained

almost neglected, notwithstanding some exceptions in freshwater (Zoppini and Marxsen 2010, Pohlon et al. 2013, Hill et al. 2017) and estuarine environments (Middelburg et al. 1996; Danovaro et al. 2002). The general lack of studies focusing on marine sediments represents an important knowledge gap because the sediment-water interface not only represents one of the largest interfaces on Earth (Middelburg 2018), but it is also the site where all physicochemical and biological processes become more intensive (Frankowski and Bolałek 1999). It functions, in fact, as a key interface in the Earth system since the processes that take place there determine whether organisms' remains are recycled within the biosphere (short-term cycle) or transferred to the geosphere (long-term cycle) (Middelburg 2018). Microbial-mediated processes in the sediments acquire, therefore, a very important role from a biogeochemical point of view. This highlights the need to study the potential influence of temperature and substrate availability/composition on benthic microbial degradative activities and C uptake. The issue is particularly pressing nowadays because understanding microbial dynamics dependence from temperature and substrates may help to detect any biogeochemical alteration due to large-scale, Climate Change-related events.

In the present study, the rates of HCP and of four benthic exoenzymatic activities (β-glucosidase, lipase, chitinase and leucine aminopeptidase) were measured over a time span of eight years (2010–2018) in order to untangle whether the bottom temperature and organic matter amount and composition acted as forcing factors on microbial metabolism and to what extent. The study was carried out at the sediment-water interface of the shallow coastal station C1 (northern Adriatic Sea), which is part of the Long Term Ecological Research-LTER network. The investigation of the same station over time allowed us to discard any attribution of any observed metabolic variation to environmental variables different from temperature and organic matter, as variations of the bathymetry and of the sediment grain size.

Materials and methods

Study site

The Gulf of Trieste is the northernmost part of the Adriatic Sea with a surface area of about 600 km^2 (Olivotti et al. 1986). Almost completely landlocked, this shallow basin (average depth of 17 m, Celio et al. 2002) is isolated from the rest of the Adriatic by a sill (~22 m depth) between Grado and the Salvore peninsula (Ogorelec et al. 1991). Freshwater inputs, which come mainly from the Isonzo River, show a high interannual variability (Comici and Bussani 2007) which affects salinity whose values range from 29 to 38 at the surface (Malačič et al. 2006). The Gulf experiences annual fluctuations of temperature (from 5 °C to ≥ 24 °C at the surface and from 6 °C to ≥ 20 °C at the bottom) and the water column is usually stratified during summer. A high variability of the water column profile, due to both riverine outflow and water temperature, is enhanced by an alternation between strong cold winds from the east-north east (i.e. Bora)

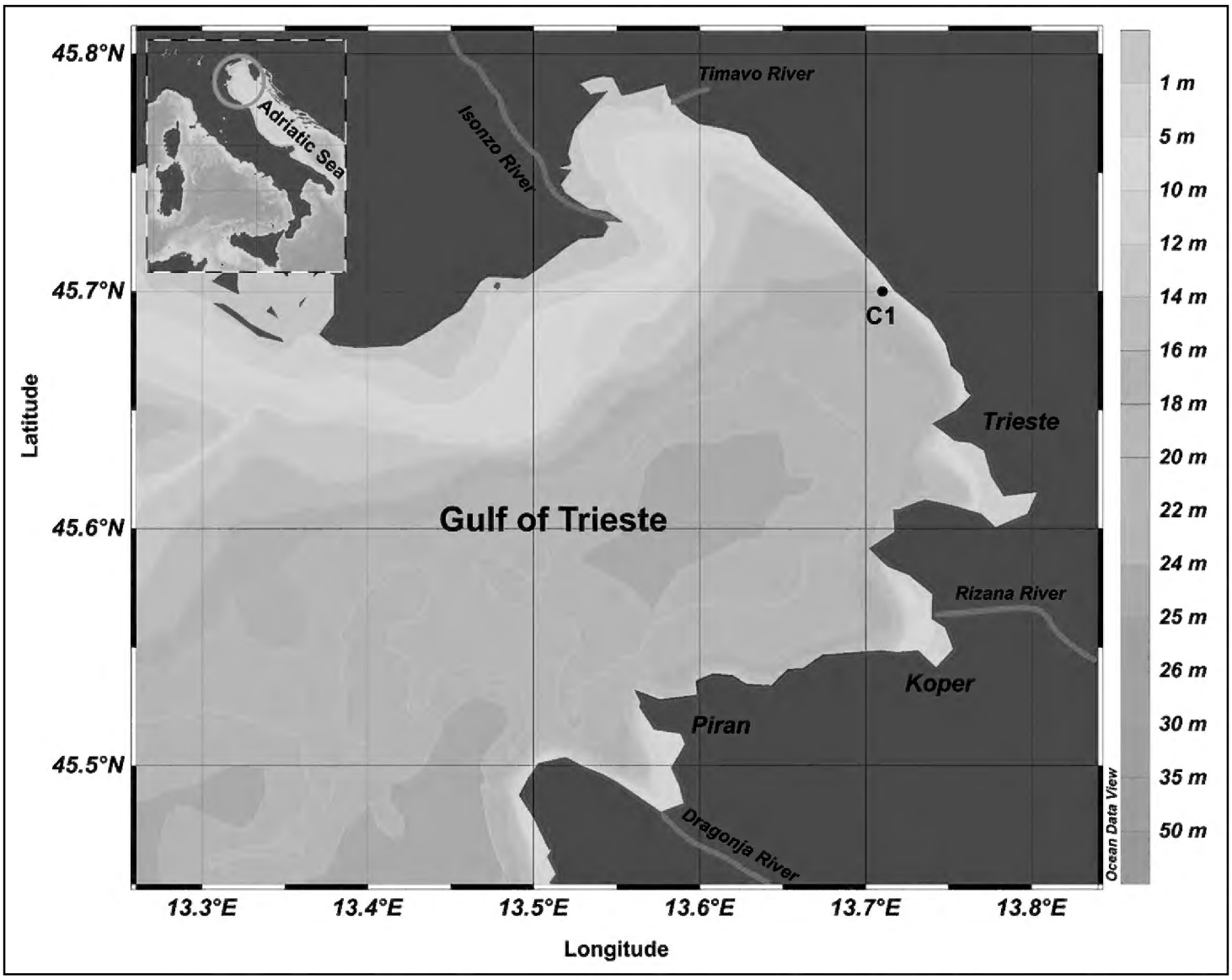

Figure 1. Location of the St. C1 (45°42'03"N, 13°42'36"E). This LTER site is nearby the outer border of the Marine Protected Area of Miramare, along the Italian coastline of the Gulf of Trieste (northern Adriatic Sea).

and mild winds from the south (i.e. Scirocco) (Lipizer et al. 2011). Sedimentation is mainly controlled by river inputs rather than by marine currents (Brambati and Catani 1988). The annual average sedimentation rate is about 1 mm yr^{-1} in the middle of the gulf and increases to 2.5 mm yr^{-1} in front of the Isonzo mouth (Covelli et al. 1999 and references therein). The sediments are mainly sandy-mud although soft bottoms can vary from sands with patches of rocks to detrital mud (Brambati and Catani 1988).

The study was carried out at the station LTER-C1, located ca. 200 m offshore (45°42'03"N, 13°42'36"E) at a depth of around 18 m, nearby the outer border of the Marine Protected Area (MPA) of Miramare (Figure 1). This small MPA is divided into two distinct zones: the inner part (30 ha), subjected to a regime of integral protection (i.e. all human activities are banned with the exception of a little corridor for diving) and a surrounding buffer zone (90 ha), sheltered by boats and professional fishing.

Sampling

From April 2010 to April 2018, sampling campaigns were performed every 3–4 months. During each sampling, the bottom sea temperature was measured using a Seabird 19 Plus-

Seacat probe. The bottom water was sampled by means of a 5-L Niskin bottle. Three virtually undisturbed sediment cores were taken by a KC Haps bottom corer (KC-Denmark, Silkeborg, Denmark) using polycarbonate sample tubes (13.3 cm I.D. resulting in a sampling surface of 127 cm^2). The sediments were collected within an experimental area of max 40 m^2. Once in the laboratory, the cores were partially extruded and the oxic sediment layer (0–1 cm ca) of each core was collected, homogenised and subsampled for all the analyses.

Total Organic C (TOC), Total Nitrogen (TN) and Biopolymeric C (BPC)

For TOC and TN analyses, sediment was freeze-dried, grounded in a ceramic mortar and then sieved on a 250 μm iron steel sieve (Endecotts LTD, UK). Triplicate subsamples of about 8–12 mg were weighed on a microultrabalance with an accuracy of 0.1 μg, directly into silver and tin capsules for TOC and TN, respectively. Before TOC determination, subsamples were treated directly into capsules with increasing concentrations of HCl (0.1 N and 1 N) to remove the carbonate fraction (Nieuwenhuize et al. 1994). Carbon and nitrogen contents were determined using a CHNO-S elemental analyzer (mod. ECS 4010, Costech, Italy) according to Pella and Colombo (1973). Standard acetanilide (Costech, purity ≥ 99.5%) were used to calibrate the instrument and empty capsules were also analyzed in order to correct for blank. Quality control of measurements was performed using internal standards and it was also verified for carbon against the certified marine sediment reference material PACS-2 (National Research Council Canada). The relative standard deviations for three replicates determination were lower than 3%. TOC and TN concentrations are expressed as mg C g^{-1} d.w. and mg N g^{-1} d.w., respectively.

For BPC analyses, subsamples of homogenised sediment were freeze-dried and processed in triplicates for the determination of carbohydrates, lipids and proteins. Colloidal and EDTA extractable carbohydrates (CHO-H_2O and CHO-EDTA, respectively) were analyzed following the method described by Blasutto et al. (2005). Lipids (LIP) were analyzed according to Bligh and Dyer (1959) while proteins (PRT) were determined following Hartree (1972). The concentrations of CHO, PRT and LIP were expressed as glucose, bovine serum albumin and tripalmitine equivalents, respectively. Data were converted to carbon equivalents using the conversion factors proposed by Fichez (1991): 0.45 for CHO, 0.50 for PRT and 0.75 g C g^{-1} for LIP.

Heterotrophic C Production (HCP)

Heterotrophic C Production (HCP) rates were carried out following the method of ^{3}H-leucine uptake for sediment samples (van Duyl and Kop 1994, as detailed by Franzo et al. 2016). Each sediment sample (0.2 mL of 1:1 v/v slurry) was added to 6 μCi of ^{3}H-leucine (Perkin Elmer) and incubated in the dark for 1 h at *in situ* temperature. After incubation, radiotracer incorporation was stopped by adding 80% ethanol (1.7 mL). After two washes of the samples with ethanol (80%) by mixing, centrifuging and supernatant removal, the sediment was transferred with ethanol (80%) onto

a polycarbonate filter (0.2 μm mesh size). Subsequently, the filters were washed twice with 5% trichloroacetic acid. Samples were heated in 2M NaOH for 2 h in a water bath at 100 °C, cooled on ice and centrifuged at 425 g for 3 minutes. One mL of supernatant was transferred to scintillation vials and 10 mL of Hionic Fluor (Perkin Elmer) scintillation fluid was added. For each sample, three replicates and two ethanol-treated blanks were analyzed. Activity in the samples was determined by a β-counter (Packard TRI-CARB 2900TR Liquid Scintillation Analyzer).

Exoenzymatic activities

Extracellular enzymatic activities were assayed using fluorogenic substrate analogues (Hoppe 1993) derived from 7-amino-4-methyl-coumarin (AMC) and 4-methyl-umbelliferone (MUF). Leucine aminopeptidase activity was assayed as the hydrolysis rate of leucine-AMC. β-glucosidase, lipase and chitinase were assayed using MUF-β-D-glucoside, MUF-oleate and MUF-*N*-acetyl-β-D-glucosaminide, respectively. Enzyme activities were expressed in terms of the rate of MUF or AMC production. Sediment slurries were prepared by adding 6 mL of 0.2 μm-filtered bottom water to 0.5 g of wet sediment. After the evaluation of saturating concentrations performed during the first sampling, hydrolysis rates were measured by incubating slurries with (final concentrations) 800-μM MUF-β-D-glucoside, 400-μM leucine-AMC and 200-μM MUF-oleate and MUF-*N*-acetyl-β-D-glucosaminide (Sigma) for 1 h in the dark and at *in situ* temperature. Before spectrofluorometric measurement, each sample was centrifuged 2 min at 1008 *g*. Fluorescence increase due to MUF and AMC hydrolysed from the model substrates was measured using a Shimadtzu RF-1501 spectrofluorometer (MUF = 365-nm excitation and 455-nm emission; AMC = 380-nm excitation and 440-nm emission). Standard solutions of MUF and AMC were used to produce calibration curves with 0.2 μm-filtered bottom water. Triplicate blanks without fluorogenic substrate were used to determine the natural fluorescence increase in the samples not attributable to the tested enzymes.

Arrhenius plots

The Arrhenius-type relationship (linearity of a natural logarithm vs inverse absolute temperature) between temperature and microbial activities was tested assuming that chemical kinetics controls the observed rates. According to the Arrhenius law, the temperature sensitivity of each exoenzymatic activity and of HCP is defined by:

$$\text{Microbial Activity} = A \cdot e^{-Ea/R*T}$$

where A is the theoretical microbial activity in the absence of the Ea; Ea is the Activation Energy, i.e. the energy barrier to be surpassed in order for the reaction to take place (in J mol^{-1}); R is the universal gas constant (8.314 J mol^{-1} K^{-1}); T is the temperature in Kelvin (K). The factor $e^{-Ea}/R \cdot T$ is proportional to the fraction of substrate molecules

with kinetic energies in excess of Ea (Arrhenius 1889). An estimate of Ea can be derived from the slope of an Arrhenius plot, which is built on the natural logarithm of each microbial activity against the inverse absolute temperature (1/T). The Ea can thereafter be calculated by multiplying the regression slope by *R*. Strictly, the Arrhenius law should be applied to a well-defined enzymatic reaction with a constant Ea and with temperature as the only factor affecting the rate. Since the organic matter consists of myriads of compounds degraded by a wide variety of microbial populations, the temperature response measured with this approach is actually the sum of all processes involved. Therefore, the calculated Ea should be seen as an apparent Ea (Middelburg et al. 1996).

Linear regression analyses were used for the Arrhenius plots. Prior to regressions, normality was checked and the confidence level was set at 95% using STATISTICA7.

Statistical analyses

A stepwise multiple regression analysis was performed in order to establish the relationship between microbial activities (HCP, β-glucosidase, lipase, chitinase and leucine-aminopeptidase) and environmental variables (temperature, TOC, TN, CHO-H_2O, CHO-EDTA, PRT and LIP). All non-normally distributed data series were log(10)-transformed before running the analysis.

In order to highlight any overall temporal pattern of microbial activities at the station LTER-C1, a cluster analysis was performed on HCP, β-glucosidase, lipase, chitinase and leucine aminopeptidase rates. The Euclidean distance and the complete linkage were applied and data were log10(x+1) transformed and normalized prior to analysis. Afterwards, the Mann-Whitney test was applied in order to test for significant differences of all microbial and environmental variables among the cluster groups. Only statistically significant results are presented.

The Spearman-Rank correlation analysis was performed to test the linear relationship between TOC and TN. Correlation coefficients (r) were considered significant at *p*-values less than 0.05.

All statistical analyses were performed using STATISTICA7 with the exception of the cluster analysis that was conducted with PRIMER software v5 (PRIMER-E Ldt, Plymouth, UK).

Results

Ranging between 7.5 °C in Mar12 and 22 °C in Jul16 (Table 1), the bottom temperature followed a seasonal pattern throughout the 8-year study, with higher values during summer samplings (June-September) and colder conditions during winter-early spring (December-March). TN concentrations varied between 1.11 ± 0.04 and 1.76 ± 0.06 mg N g^{-1} (Dec16 and Feb11, respectively) while TOC ranged from 9.85 ± 0.23 to 18.09 ± 0.14 mg C g^{-1} (in Dec16 and Jul10, respectively). Although both variables showed a certain temporal variability, overall higher amounts characterized sum-

Table 1. Environmental variables measured at the sea bottom (Temp = temperature) or in sediment surface layer. CHO-H2O =colloidal carbohydrates; CHO-EDTA = Carbohydrates extracted in EDTA; PRT = proteins; LIP = lipids; n.a. = not available. With the exception of Temp, values are averages of 3 replicates.

	Temp	CHO-H2O			CHO-EDTA			PRT			LIP		
	°C	μg C g^{-1}			μg C g^{-1}			μg C g^{-1}			μg C g^{-1}		
Apr10	11.18	70.10	±	1.56	149.92	±	6.93	557.49	±	29.55	626.36	±	30.02
Jul10	16.53	27.48	±	1.10	170.18	±	2.77	627.84	±	26.79	1069.42	±	43.39
Nov10	15.50	71.27	±	2.19	141.35	±	4.26	590.21	±	24.23	937.49	±	51.83
Feb11	8.71	43.04	±	0.13	184.81	±	5.85	426.90	±	15.75	859.93	±	33.14
Jun11	15.12	95.04	±	3.52	285.17	±	17.05	957.12	±	13.33	1192.15	±	8.77
Sep11	19.42	85.24	±	3.67	219.45	±	5.95	888.92	±	38.09	1328.65	±	2.08
Dec11	12.72	60.08	±	1.49	206.73	±	6.51	881.94	±	11.34	976.34	±	23.38
Mar12	7.47	85.14	±	5.31	97.01	±	5.07	1109.63	±	26.85	835.08	±	6.09
Jul12	19.13	85.45	±	5.23	347.14	±	3.90	1123.10	±	27.13	1134.46	±	54.83
Nov12	15.00	n.a.			n.a.			n.a.			n.a.		
Feb13	9.00	108.25	±	4.67	140.14	±	1.96	512.84	±	20.86	769.26	±	31.29
Apr13	9.50	164.02	±	2.36	267.04	±	16.11	639.92	±	29.61	959.88	±	44.41
Nov13	16.60	110.82	±	4.75	312.71	±	15.07	637.75	±	17.55	956.62	±	26.33
Apr14	13.50	112.60	±	8.82	177.39	±	8.13	574.66	±	13.30	861.99	±	19.96
Jul14	18.20	115.73	±	3.12	93.83	±	4.45	573.84	±	28.98	860.75	±	43.47
Mar15	10.00	87.49	±	2.19	225.99	±	12.70	691.42	±	13.71	823.81	±	50.11
Jun15	19.50	144.25	±	0.35	274.63	±	10.11	444.93	±	19.48	954.55	±	55.66
Oct15	17.40	76.13	±	2.14	253.43	±	1.35	613.61	±	10.57	748.53	±	26.94
Jan16	9.45	93.06	±	3.36	267.29	±	6.12	322.10	±	18.81	1067.77	±	30.71
Apr16	13.10	102.60	±	1.16	308.67	±	19.61	316.30	±	14.38	662.53	±	30.14
Jul16	22.00	129.87	±	2.10	325.18	±	8.49	462.49	±	30.21	921.30	±	34.09
Oct16	18.70	119.41	±	6.30	94.38	±	2.53	419.14	±	23.58	705.92	±	37.81
Dec16	13.00	47.67	±	2.08	274.07	±	4.33	231.85	±	7.90	447.87	±	78.07
Apr17	11.40	139.45	±	4.51	437.44	±	21.64	321.30	±	16.38	686.25	±	39.41
Jul17	17.70	147.92	±	1.59	420.28	±	1.10	470.87	±	24.20	479.54	±	30.24
Oct17	19.20	96.40	±	2.49	411.50	±	6.10	385.77	±	0.90	581.62	±	22.67
Jan18	10.90	80.76	±	0.59	386.35	±	11.10	295.40	±	10.21	759.16	±	55.64
Apr18	10.20	133.16	±	2.41	482.11	±	20.69	511.04	±	40.92	822.06	±	22.47

mer samplings while lower ones were observed mainly during winter (Figure 2). With concentrations between 27.48 ± 1.10 (Jul10) and 164.02 ± 2.35 μg C g^{-1} (Apr13), colloidal carbohydrates (CHO-H_2O) represented the minor fraction of the BPC pool throughout the entire study (Table 1). While minima of carbohydrates extracted in EDTA were comparable to CHO-H_2O (~100 μg C g^{-1}), overall this biopolymeric fraction showed more elevated concentrations, especially from April 2017. During the last five samplings (2017–2018), in fact, CHO-EDTA accounted for values >386.35 ± 11.10 μg C g^{-1} reaching the highest concentrations of the whole study period (Table 1). Representing on average 31% of the BPC pool, proteins were the second dominant fraction after lipids. Notwithstanding, proteinaceus material was clearly more abundant in 2011–2012 samplings while overall lower amounts were observed from January 2016 (Table 1). Lipids accounted on average for 48% of BPC and the values ranged from 447.87 ± 78.08 to 1328.65 ± 2.08 μg C g^{-1} in Dec16 and Sep12, respectively (Table 1). A clear seasonal pattern, e.g. higher values during summer and lower ones during winter, was observed for none of the four biopolymeric fractions considered.

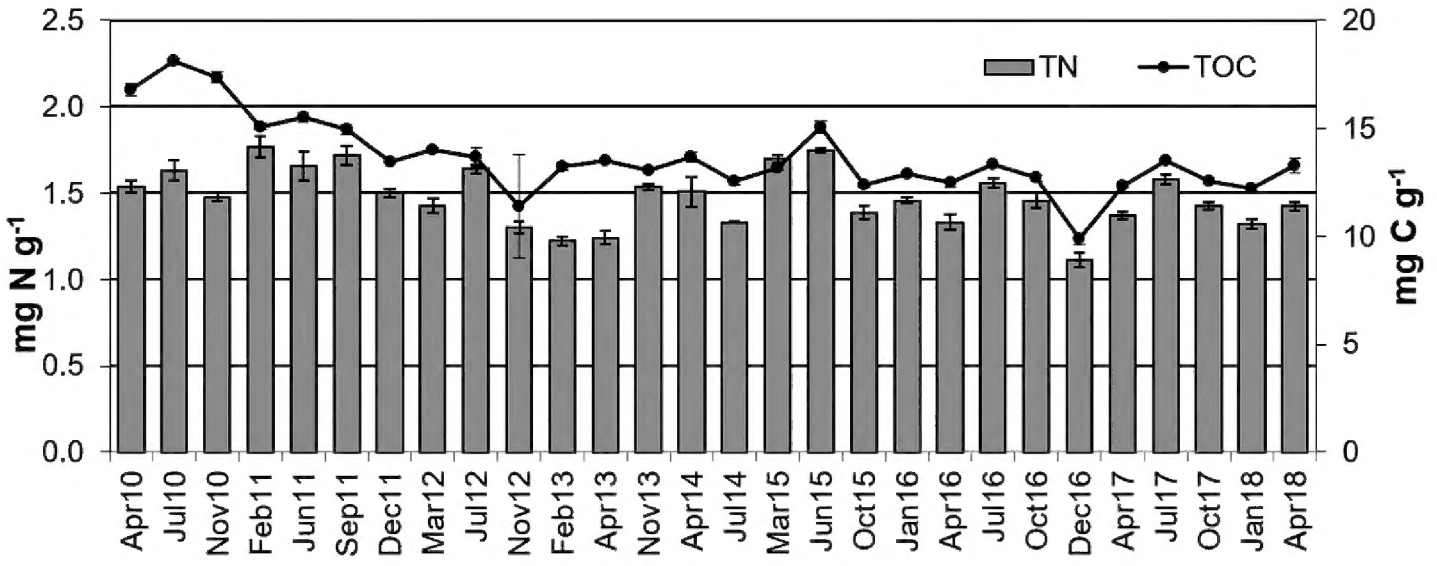

Figure 2. Mean concentrations of TN and TOC (±SD) over the 8-year study.

Minima of HCP rates characterized winter samplings (Nov12 and Dec16) while maxima were measured in spring and early-summer (Apr16 and June15) (Figure 3a). The degradation of polysaccharides was more pronounced in summer and early-autumn samplings. This was more evident in Jun11 and Sep11 when the fastest hydrolytic rates were measured (~17 nmol cm^{-3} h^{-1}) while, throughout the entire study, velocities > 5 nmol cm^{-3} h^{-1} were observed in June, July or October with the exception of Apr13 (Figure 3b). With an average rate of ~4 nmol cm^{-3} h^{-1}, the degradation of lipids was the less pronounced microbial activity in the sediments of the station LTER-C1 during the 8-year study. Notwithstanding, similarly to β-glucosidase, the rates were >5 nmol cm^{-3} h^{-1} only in June, July, September and October and reached peaks equal to 16.94 ± 1.43 and 12.18 ± 0.85 nmol cm^{-3} h^{-1} in Jul12 and Jun15. On the contrary, values lower than 1 nmol cm^{-3} h^{-1} were measured in Mar15, Apr16 and Jan18 (Figure 3c). Focusing on the degradation of chitin, two evident maxima were observed in Jun11 (32.99 ± 0.81 nmol cm^{-3} h^{-1}) and in Jul17 (16.95 ± 0.33 nmol cm^{-3} h^{-1}). Similarly to the hydrolytic activities described above, chitinase rates resulted >5 nmol cm^{-3} h^{-1} in June, July, September and October while lower values characterized the remaining samplings (Figure 3d). In the present study, the highest hydrolitic activity was addressed to the degradation of polypeptides since the measured rates resulted in at least 1 order of magnitude faster than those ascribed to the other exoenzymes considered. With the exclusion of the highest value measured in Jun11 (568.59 ± 24.14 nmol cm^{-3} h^{-1}), summer and early-autumn samplings showed velocities comparable with those observed during early-spring (March and April) (Figure 3e).

Significant temperature-dependences were described by Arrhenius-type equations for HCP and all the degradative activities tested with the exception of leucine-aminopeptidase (Figure 4). The calculated Ea was lower for HCP rates (29.27 kJ mol^{-1}) and overall higher for the degradation of the main macromolecules according to the following order: chitin (55.61 kJ mol^{-1}), polysaccharides (63.99 kJ mol^{-1}) and lipids (75.21 kJ mol^{-1}).

The stepwise multiple regression analysis confirmed the influence of the bottom temperature on both the microbial C uptake and the majority of the tested exoenzymatic activities. The only exception was represented by the aminopeptidase since it was

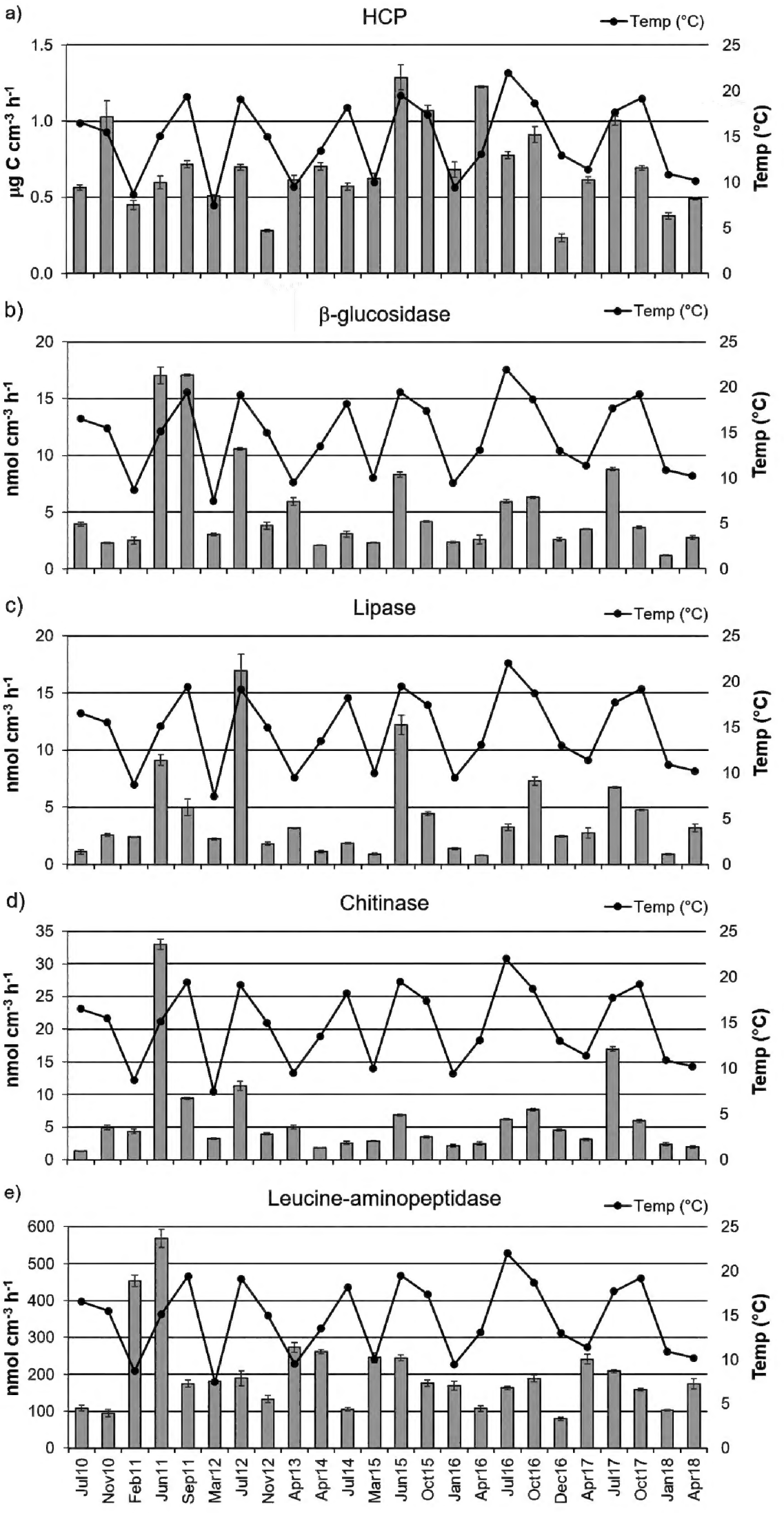

Figure 3. Rates of the tested microbial activities from July 2010 to April 2018.

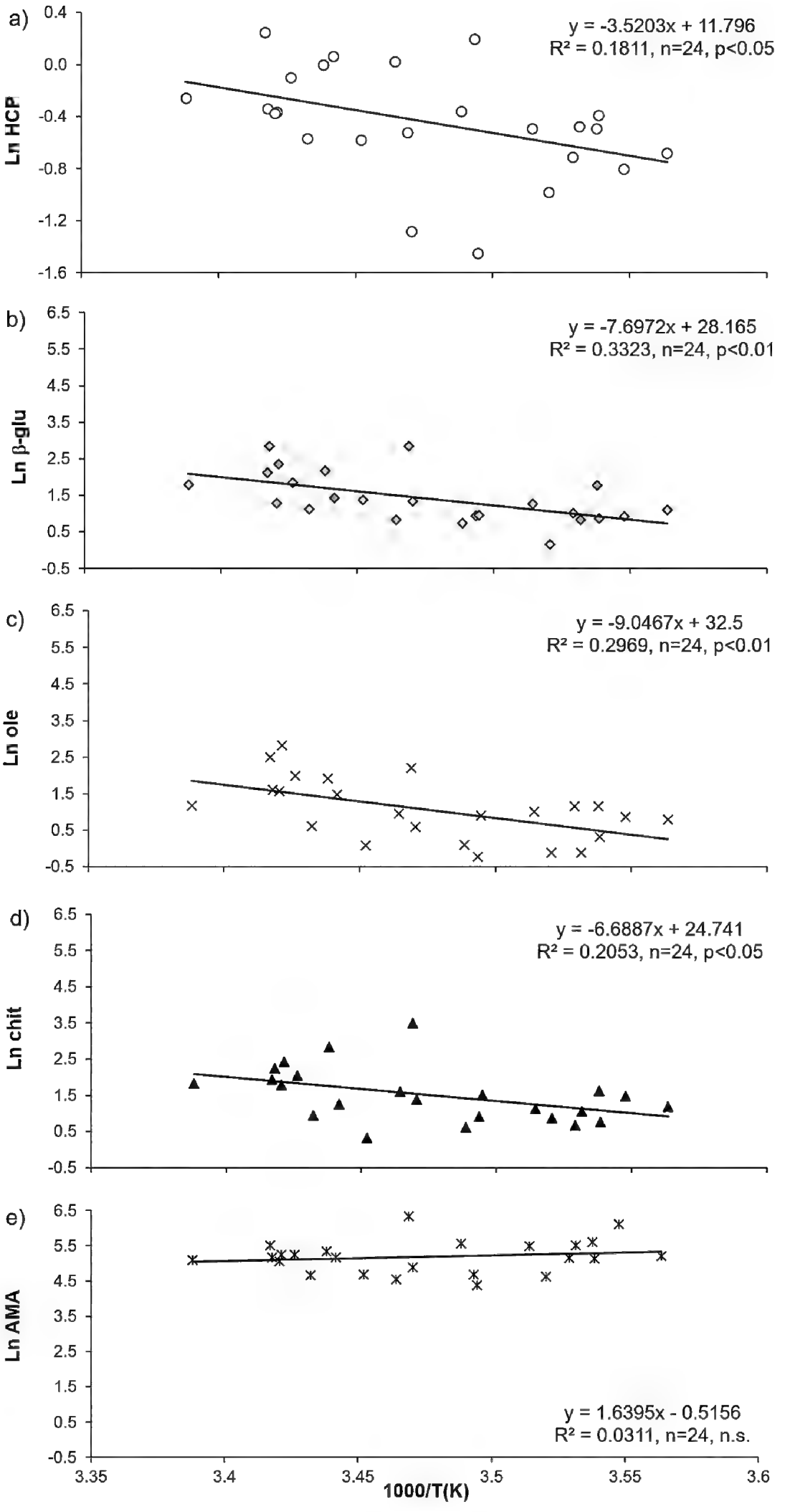

Figure 4. Arrhenius plots of the natural logarithm of each microbial activity against the inverse absolute temperature (1/T). HCP = Heterotrophic C Production (**a**); β-glu = β-glucosidase (**b**); ole = lipase (**c**); chit = chitinase (**d**); AMA = leucine aminopeptidase (**e**); n.s.= not significant.

Table 2. Stepwise multiple regression analysis between microbial activities and environmental variables (*p<0.05; **p<0.01; ***p<0.001). HCP = Heterotrophic C Production; AMA = Leucine aminopeptidase activity; Temp = bottom temperature; TOC = Total Organic C; TN = Total Nitrogen; CHO-H2O =colloidal carbohydrates; CHO-EDTA = carbohydrates extracted in EDTA; LIP = Lipids; PRT = proteins.

	F	Adj r^2	n	Variables
HCP	3.410*	0.30	23	Temp* CHO-H2O TOC LIP
β-glucosidase	6.115**	0.54	23	Temp** PRT CHO-H2O LIP CHO-EDTA
Lipase	5.584**	0.38	23	Temp** PRT* CHO-EDTA
Chitinase	3.275*	0.24	23	Temp* PRT CHO-EDTA
AMA	8.359***	0.50	23	TN*** CHO-H2O* Temp*

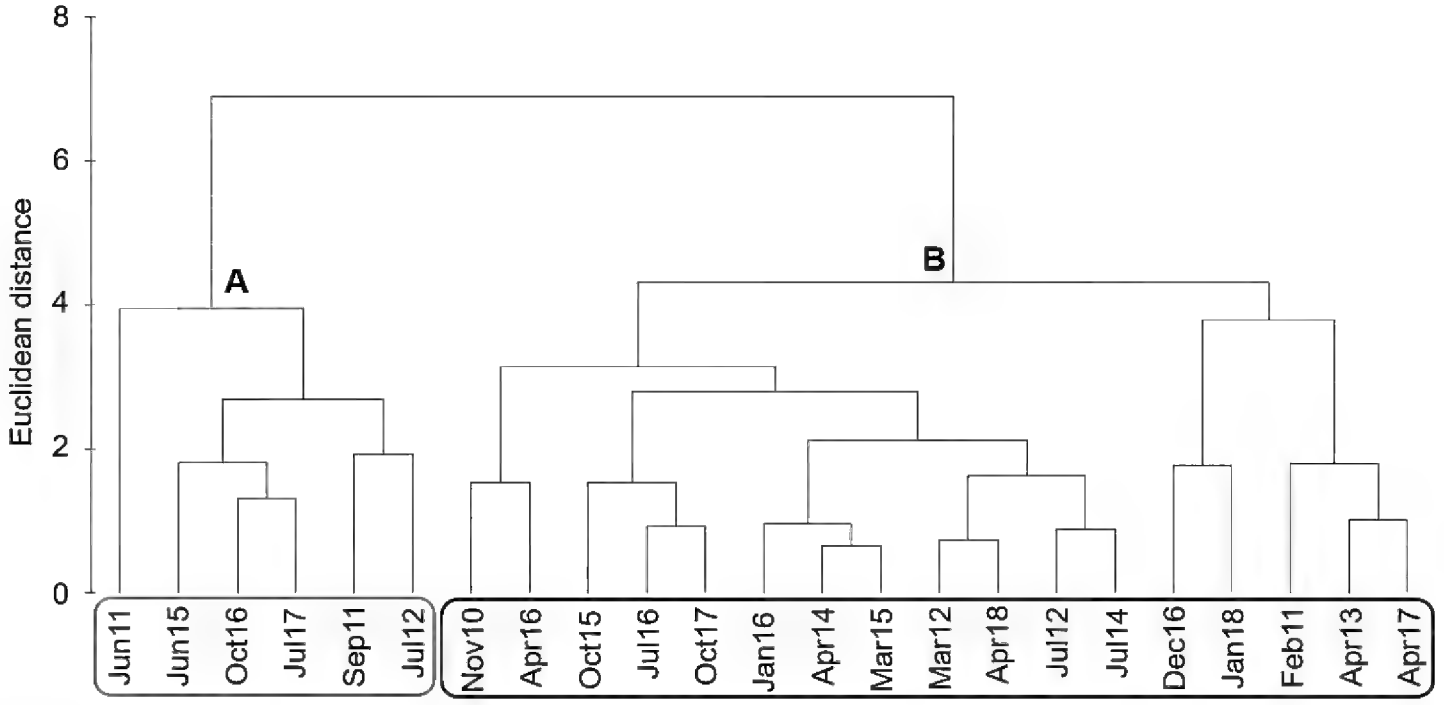

Figure 5. Cluster analysis performed on the tested microbial activities. Euclidean distance and the complete linkage were applied.

explained for 50% by N-containing material, expressed in terms of TN, and, to a lesser extent, by colloidal carbohydrates and bottom temperature (Table 2).

The cluster analysis performed on the tested microbial activities clearly separated the majority of summer and early-autumn samplings (Group A) from the remaining observations (Group B; Figure 5). The Mann-Whitney test pointed out that group A

gathered samplings with significantly faster rates of β-glucosidase, lipase and chitinase ($Z=3.57$, $p<0.001$) than those belonging to group B. On the contrary, HCP and protease velocities were not statistically different between the two groups identified. Focusing on the environmental variables, the Mann-Whitney test indicated that only the bottom temperature ($Z=2.52$, $p<0.05$) and TN ($Z=2.45$, $p<0.05$) significantly differed between A and B, i.e. higher values for the former group and lower ones for the latter.

Discussion

Over a time span of eight years (2010–2018), five benthic microbial processes - i.e. HCP, β-glucosidase, lipase, chitinase and aminopeptidase - were related to the temporal fluctuations of the bottom temperature and to the variable amount and composition of sedimentary organic matter. Overall, the fastest rates of both organic matter degradation and microbial growth were observed in warmer conditions while a limited C reworking was detected at lower temperatures. This is a general pattern already observed in the water column (Arnosti et al. 2011, Calvo-Díaz et al. 2014) and at the sediment-water interface (Perliński and Mudryk 2018). Arnosti et al. (2011) suggested the existence of a latitudinal gradient for the degradation of marine dissolved organic carbon: the warm superficial waters of tropical and subtropical areas showed faster degradative rates than their cold counterpart at higher latitudes. Focusing on the effects of temperature temporal fluctuations on microbial metabolism, Perliński and Mudryk (2018) measured the highest hydrolytic activities during the vegetation period (spring, summer and autumn) at the sediment-water interface along a coastal river estuary. Similarly, a pronounced seasonal variability was reported in the water column also at St. C1, with winter minima and maxima from April to October for six exoenzymatic activities measured on a monthly basis between 2000 and 2005 (Celussi and Del Negro 2012). As shown by our data, analogous empirical evidences characterized also the sediments of this site and were statistically validated by the Mann-Whitney outputs and by the cluster analysis. Throughout the study period, in fact, summer and early-autumn samplings, characterized by overall faster microbial activities (group A), gave results that were significantly separated from those in group B, which gathered samplings with overall low (e.g. Nov12 and Dec16) and intermediate velocities (i.e. Apr13, Marc15 and Apr17). Furthermore, the significant correlations between the bottom temperature and the majority of the tested processes pointed out in the Arrhenius plots supported the dependence of the microbial metabolism from this environmental variable.

The Arrhenius plots describe a single microbial-mediated reaction that is performed on a homogeneous substrate in terms of composition and bioavailability. Undoubtedly this is an ideal situation that does not occur in natural environments since the bulk of organic material is composed by a wide plethora of molecules, each characterized by a dissimilar composition and, therefore, by a different degree of complexity (Burdige 2006). Notwithstanding, in the present study the outputs of the stepwise correlation analysis confirmed the dependence of the microbial metabolism from the bottom tem-

perature for all the tested activities. While very little is known about marine sediments, temperature sensitivity of organic matter degradation by microbes has been studied extensively in soils (Davison and Janssens 2006; Davison et al. 2012; Sierra 2012). The carbon quality temperature hypothesis (CQT) is based on the principles of enzyme kinetics combined with the Arrhenius law. According to CQT, substrate quality can be defined by changes in Ea, i.e. the more recalcitrant the substrate, the higher its corresponding Ea (Sierra 2012). In the present study, Ea values (29.27- 75.21 kJ mol^{-1}) were similar to those calculated for organic matter degradation in marine sediments (Middelburg et al. 1996) and for heterotrophic prokaryotic production in the epipelagic and the mesopelagic (Lønborg et al. 2016). Although the highest Ea measured during this 8-year study implies a somewhat more recalcitrant nature of sedimentary lipids, this value was lower than that proposed for the degradation of actual recalcitrant organic matter in marine sediments (~ 200 kJ mol^{-1}; Burdige 2011), suggesting that at St. C1 microbial-accessible substrates were available to benthic prokaryotes over the entire study period. The Ea calculated for HCP further corroborated this hypothesis. Its value was, in fact, clearly lower than the theoretical Ea of 62.7 kJ mol^{-1}, which indicates the energy for the overall heterotrophic metabolism if the temperature were the only factor involved. The consequent implication is that environmental factors other than temperature likely influenced prokaryotic C uptake at St. C1 (e.g. the organic matter pool) over the entire study period (Brown et al. 2004; López-Urrutia and Morán 2007). During this 8-year time span, benthic prokaryotes likely benefited from favorable conditions of substrate availability that may have supported the observed HCP rates by lowering the energy required for activating this process.

In the present study, the degradation of proteins was the only exoenzymatic activity that resulted weakly temperature-dependent, as indicated by the lack of significance in the corresponding Arrhenius plot. On the other hand, this hydrolytic activity resulted mainly related to the substrate availability, expressed in terms of TN. In coastal sediments, this pool comprises a wide spectrum of inorganic and organic compounds, with the former fraction mainly represented by fixed ammonium ions entrapped in illite crystal structure (Schubert and Calvert 2001). At St. C1, this inorganic fraction cannot be excluded since its amount was reported to not exceed ~20% of the whole pool (Faganeli et al. 1991). Nevertheless, TOC (wt %) and TN (wt %) resulted tightly correlated (n=28, r=0.67; $p<1\times10^{-5}$) as reported also by Rumolo et al. (2011) in the harbour of Naples (Southern Tyrrhenian Sea). As inferred by these authors, this strong correlation allows us to reasonably assume that TN concentrations measured at St. C1 were mainly organic (Rumolo et al. 2011).

In the Gulf of Trieste, a preferential decomposition of organic nitrogen and total phosphorous with respect to organic carbon was suggested by Faganeli et al. (1991) as a result of a stable isotope study on marine sediments. The stepwise correlation analysis in the present study further confirmed this overall pattern since TN was indicated as the environmental driver that mainly influenced the most pronounced hydrolytic activity observed, i.e. the degradation of polypeptides. Despite the lack of significant correlation between aminopeptidase and bottom temperature, also this exoenzymatic

activity showed an overall temporal pattern with faster rates in summer samplings. This pattern mirrored mainly seasonal TN fluctuations rather than temperature, posing the question about the possible environmental drivers responsible for TN temporal variability. In coastal areas where the euphotic zone extends to the sediment-water interface, benthic microalgae are known for producing readily-usable organic carbon (Welker et al. 2002 and references therein). Accordingly, Hardison et al. (2013) demonstrated in a mesocosm study that microphytobenthos, and in particular benthic diatoms, determined a sensible increase of both TN and sediment organic matter (SOM) not only in terms of amount but also of lability. The latter was investigated by means of a suite of Specific Total Hydrolysable Amino Acids (THAA) and Phospholipids Linked Fatty Acids (PLFA) for which it was possible to ascribe the respective major organic matter sources. During the experiment time-course, the authors observed that the increase of TN and SOM was mainly due to the PLFA attributable to diatoms, demonstrating therefore the role of these microalgae in contributing to the lability of the organic carbon pool. Furthermore, the observed increase of PLFA concentrations of bacterial origin indicated that diatom proliferation supported the heterotrophic bacterial community, highlighting an important link between these two assemblages. The shallow depth of St. C1 allows the solar radiation to reach the seafloor and, consequently, the growth of an active and abundant community of microalgae is promoted (Welker et al. 2002, Cibic et al. 2007). Seasonal peaks of abundance during summer have been already reported and ascribed to favorable conditions of irradiance and temperature (Welker et al. 2002, Cibic et al. 2012). Considering the period 2010–2018, such peaks of abundance have been documented during the first two years (Franzo et al. 2016) and were observed also afterwards (Cibic, personal communication). The proliferation of benthic microalgae may have contributed not only to the observed TN peaks but also to an increased lability of the substrates fueling, therefore, microbial-mediated processes and in particular those oriented to the degradation of polypeptides. The future implementation of the study of microbial metabolism with microphytobenthic data would allow the exploration of the interactions between these two benthic assemblages mediated by palatable organic material of photosynthetic origin.

Conclusions

Over a time span relatively long for data of microbial-mediated processes, this study allowed us to understand how two environmental factors – i.e. temperature and substrate availability – determine the microbial C-cycling in temperate shallow marine sediments. Similar to the soils and the water column, a clear dependence of prokaryotic activities from temperature was confirmed at the sediment-water interface, a biologically active interface largely neglected despite its ecological importance.

Overall, relatively low Ea values were measured, suggesting that the availability of microbial-accessible substrates to prokaryotes was not negligible throughout the study period. In particular, the presence of palatable substrates likely lowered the Ea required

for HCP, resulting, in fact, in a value clearly lower than that theoretically required if the temperature were the only factor involved.

Nevertheless, a clear and tight dependence from the substrate availability was detected only for aminopeptidase, the most pronounced degradative activity observed. Identified as the substrate that mainly drove the degradation of polypeptides, TN varied temporally, posing therefore the question on what caused such fluctuations. Known for increasing TN concentration and bioavailability, benthic microalgae are suggested here as the best candidates. Future implementations with detailed macromolecular fingerprinting of sedimentary organic matter would allow the identification of the substrates preferentially used by benthic prokaryotes and to link them definitely to their probable microalgal origin.

Our 8-year dataset demonstrated to be sufficiently robust to obtain a glimpse about the effects of temperature and substrates on shallow microbial metabolism. According to the LTER philosophy, its continuation over the years deserves to be promoted because only in a long-term time span the functional-microbial perspective would contribute in detecting possible biogeochemical deviations, especially in consideration of current global challenges as Climate Change-related events. The membership of St. C1 to LTER-Italy represents an added value for our current and future results because the network will magnify their sharing and dissemination, in accordance with the perspective of Open Science.

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